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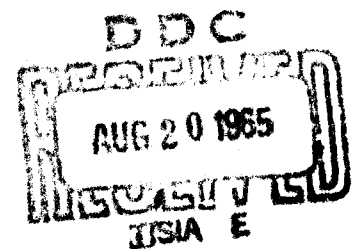
TECHNICAL MANUSCRIPT 231

**PATHOGENESIS OF THE LETHAL EFFECT
OF ANTHRAX TOXIN IN THE RAT:**

**I. PHYSIOLOGIC AND
PHARMACOLOGIC STUDIES**

Francis A. Beall
Frederic G. Dollidorf

JULY 1965



**UNITED STATES ARMY
BIOLOGICAL LABORATORIES
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PATHOGENESIS OF THE LETHAL EFFECT OF ANTHRAX TOXIN IN THE RAT:
I. PHYSIOLOGIC AND PHARMACOLOGIC STUDIES

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Project IC014501B71A01

July 1965

In conducting the research reported here, the investigators adhered to "Principles of Laboratory Animal Care" as established by the National Society for Medical Research.

ABSTRACT

The pathogenesis and nature of the pulmonary edema caused by lethal doses of anthrax toxin in Fischer 344 rats were investigated. It was shown that massive pulmonary edema developed only after a lag period during which signs of illness were absent. The protein content and electrophoretic analyses of exudates collected from rats killed by toxin indicated that plasma had escaped from the pulmonary circulation. Following injection of sublethal doses of toxin, a delayed but prolonged vascular leakage in pulmonary, peritoneal, and subcutaneous vascular beds was also demonstrated. Pulmonary edema was not prevented nor was survival time increased by treating rats with drugs inhibiting histamine or serotonin or with drugs blocking the autonomic nervous system at several sites.

II. INTRODUCTION

A previous report from this laboratory showed that rats were killed much faster by less anthrax toxin per gram of body weight than were mice or guinea pigs.¹ Clinical symptoms and postmortem observations were also described. The relatively high susceptibility of the Fischer 344 rat to the lethal effects of anthrax toxin has since been confirmed by other investigators.²⁻⁴ The present study investigates the manner in which toxin exerts its lethal effect in the rat in order that the significance of this phenomenon in the pathogenesis of anthrax may be evaluated. Most of the following experiments were concerned with the pathogenesis and nature of the pulmonary edema caused by anthrax toxin in rats.

III. MATERIALS AND METHODS

A. ANTHRAX TOXIN

The Sterne (Weybridge) strain of Bacillus anthracis was grown in caseamino acids medium under conditions previously described.¹ After 24 hours' growth, the bulk of the bacteria was removed by centrifugation and the crude or unfractionated toxin used in these experiments was prepared in one of two ways: (i) the culture was sterilized by filtration through membrane filters (Millipore®) held in stainless steel funnels, frozen, and stored at -20 C, or (ii) horse serum (10% v/v) was added to the supernatant culture fluid, which was then sterilized by passage through fritted-glass filters of ultrafine porosity, shell-frozen, lyophilized, and stored at -20 C. Horse serum stabilizes anthrax toxin to lyophilization and prevents the adsorption of toxic fractions on fritted glass. Toxin prepared as in (i) was used exclusively in the experiments demonstrating increased capillary permeability by dye leakage; preparation (ii) was used in the experiments on drug treatments. The results on increased lung weights were obtained with both preparations. These culture filtrates killed all rats given 0.4 cc or more intravenously. Injection of 2.0 cc by this route killed rats in 60 to 70 minutes and 1.0 cc killed them in 80 to 90 minutes. A portion of the culture filtrates prepared as in (i) above was subsequently passed through fritted-glass filters until toxic factors had been removed to the extent that intravenous injection of 4.0 cc of the final filtrate no longer elicited symptoms of toxicity in rats and the material had no titer in the guinea pig assay for formation of cutaneous edema.¹ This material is referred to as nontoxic culture filtrate.

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B. RATS

Male Fischer 344 rats, weighing 200 to 300 grams, were used in these experiments. Most of these came from the colony maintained at Fort Detrick⁶ and some were purchased from The Charles River Breeding Laboratories, Inc., Brookline, Mass., or from Microbiological Associates, Inc., Walkersville, Maryland. Unless otherwise stated toxin was injected into the dorsal vein of the penis. Doses of toxin up to 2.0 cc were administered by this route.

C. LUNG WEIGHTS

Lungs from rats killed by anthrax toxin and from rats sacrificed by crushing the cervical spine were weighed. The trachea was severed near the bifurcation, and the heart, esophagus, and thymus were carefully removed. The weight of the lungs was determined to 0.01 g and reported as a per cent of the body weight, which had been determined to the nearest 5 g. The term lung weight used here refers to this value.

D. PROTEIN ANALYSIS

Rat sera and exudates were analyzed for protein content by a modification of the biuret method⁷ and by means of paper electrophoresis.*

E. DRUGS AND DOSAGES

The following drug treatments were tested for effect in altering the response of rats to a lethal intravenous dose of toxin.

Nicotine was injected subcutaneously (4 mg per kg) 30 minutes before the toxin was injected and three times afterward at intervals of 20 to 30 minutes.

Priscoline[®] (tolazoline hydrochloride) - (Ciba Pharmaceutical Co.) was given intraperitoneally (48 mg per kg) 30 minutes before and after injection of toxin.

Dibenzyline[®] (phenoxybenzamine) - (Smith, Kline and French Laboratories) was injected subcutaneously (4 mg per kg) 20 minutes before the toxin and at 60 and 100 minutes afterward.

Atropine (4% atropine sulfate) - (Crookes-Barnes Laboratories) was given intraperitoneally (1 mg per kg) at 2 hours and at 1 hour before the toxin; another dose (4 mg per kg) was injected intravenously 1 hour after the toxin.

* Beckman Instruments, Model R electrophoresis system, RDM-5.

Phenergan[®] (promethazine hydrochloride) - (Wyeth Laboratories) was injected subcutaneously (25 mg per kg) 30 minutes before the toxin, and intravenously (1.6 mg per kg) 40 minutes after the toxin.

The following drugs were also used in these studies as indicated in the text: Nembutal[®] (pentobarbital sodium) - (Abbott Laboratories); Compound 48/80 (Burroughs Wellcome and Co.); Serotonin (serotonin creatinine sulfate) - (Nutritional Biochemicals Corp.); UML-491 (methysergide bimalate) - (kindly supplied by Sandoz Pharmaceuticals).

F. DEMONSTRATION OF INCREASED VASCULAR PERMEABILITY BY DYE LEAKAGE

One cc of 1% Evans blue dye was injected intravenously into rats at intervals ranging from 30 minutes to 12 hours following the administration of toxin. Five minutes after injection of Evans blue, the rats were sacrificed by intravenously injecting 30 mg of Nembutal[®]. The color of lungs from rats that had been given toxin intravenously was compared with that of lungs from rats given Evans blue alone. Following intradermal or intraperitoneal injection of toxin and nontoxic culture filtrates, the color of injection sites and peritoneal exudates was noted. Colored photographs of tissue specimens were taken and the optical density of peritoneal exudates was determined at 630 mμ.

III. RESULTS

A. DEVELOPMENT AND SEVERITY OF PULMONARY EDEMA

The extent of pulmonary edema in rats given anthrax toxin was determined by weighing the lungs. The mean lung weight of 55 normal rats was 0.59% of body weight; the standard deviation from this mean was ± 0.11 . The corresponding values obtained with 141 rats killed in 1 to 3 hours by toxin was $1.49 \pm 0.18\%$ of body weight.

In other experiments, pulmonary exudates were collected for protein analysis. As much as 2 cc of exudate was obtained from rats at death by exposing and cutting the trachea to collect the frothy fluid that emerged. In addition, from 1 to 3 cc of clear fluid could be aspirated from the opened chest cavity. Exudates coagulated upon standing and analyses were performed on fluid expressed from the clots. The mean total protein content of fluid from the trachea and from the chest cavity of 13 rats was 5.5 and 4.9 g per 100 cc, respectively. The mean value for 20 normal rat sera analyzed at the same time was 6.4 g per 100 cc. The paper electrophoresis patterns in Figure 1 are typical of those obtained with

exudates and normal sera and show that the exudates contained the major serum proteins. These observations indicate that the fluid escaping from lung capillaries of the rat, at least in the terminal stage, is essentially plasma.

To determine when pulmonary edema developed, lungs from rats sacrificed at intervals after injection of a lethal dose of toxin were weighed. Figure 2 shows the lung weights of 57 rats given a dose of toxin that killed them in 80 to 100 minutes. Lung weights were determined in 14 rats at the time of death from toxin; the remaining 43 were sacrificed at the times indicated. These results show that there is no appreciable accumulation of fluid in the lungs for about 50 minutes after the injection of this dose of toxin. From this time, lung weights increased until terminal values as high as 2% of body weight were reached. The time at which pulmonary edema was detected corresponds to the onset of clinical symptoms.

The lag in onset of pulmonary vascular leakage was also demonstrated in the following experiment. Rats were given an intravenous dose of toxin that killed them in 80 to 90 minutes. At various times thereafter, Evans blue was injected by the same route and the animals were sacrificed 5 minutes later. Lungs from animals sacrificed at 30, 40, and 45 minutes were of the same dusky gray color as lungs from rats given Evans blue only. Lungs taken at 60 minutes or later were stained blue; the intensity of the blue color increased with time after injection of toxin. In a similar experiment, Evans blue was injected and rats were sacrificed at intervals after the intravenous injection of a sublethal dose of toxin (0.25 cc). Lungs from animals sacrificed at $\frac{1}{2}$, 1, 2, and 3 hours after this dose showed no gross evidence of increased blue staining. Lungs taken at 4, 5, 6, 8, 10, and 12 hours were stained blue and were edematous.

B. EFFECT OF DRUGS ON THE RESPONSE OF RATS TO THE LETHAL EFFECTS OF ANTHRAX TOXIN

Rats were given the drugs listed in Table 1 to see whether such treatment had an inhibiting effect on pulmonary edema caused by toxin. Each of these drugs has been reported to prevent some form of experimental pulmonary edema. For example, Cameron and Sheikh⁸ prevented the formation of pulmonary edema caused by ammonium salts in the rat with nicotine and with adrenergic blocking agents. In the present experiment, rats were treated as described in Section II and littermates of these rats were given appropriate volumes of 0.9% sodium chloride solution. The large doses of drugs did not, by themselves, kill rats or affect lung weights. Rats in both groups were then given a lethal dose of toxin (usually 0.5 cc) intravenously. The results in Table 1 show that rats given drugs did not survive as long as rats given saline, and that pulmonary edema was not markedly inhibited.

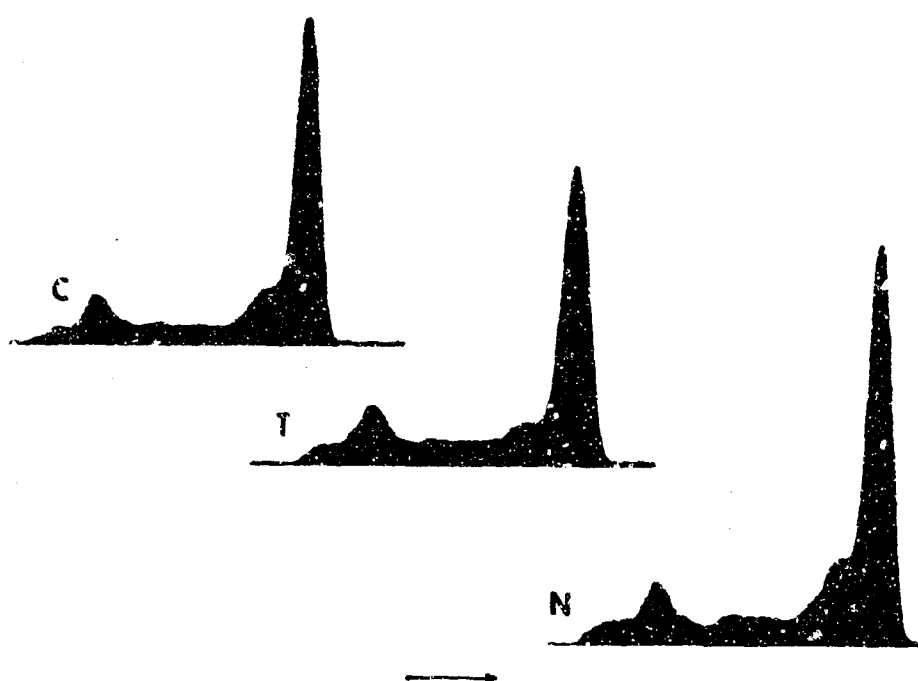


Figure 1. Electrophoretic Patterns of Normal Rat Serum (N), Edema Fluid from the Trachea (T), and from the Chest Cavity (C).

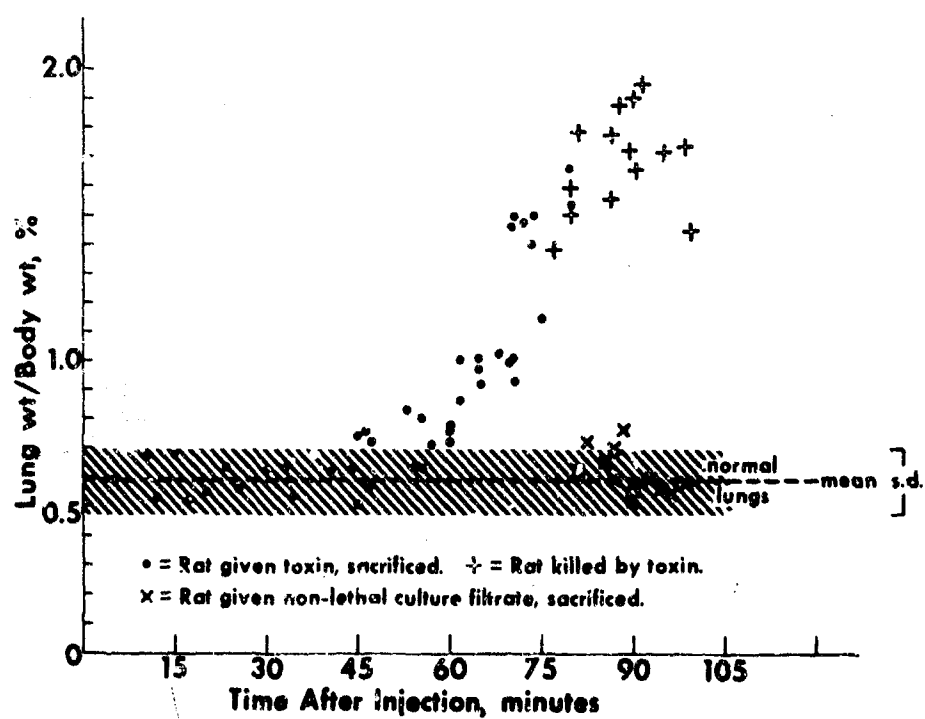


Figure 2. Development of Pulmonary Edema in Rats Given Anthrax Toxin.

TABLE 1. EFFECT OF DRUGS ON RESPONSE OF RATS
TO ANTHRAX TOXIN

No. of Rats	Treatment	Time to Death, minutes (Mean \pm SD)	Lung Weight, g. (Mean \pm SD)
10	Nicotine	140 \pm 19	1.43 \pm 0.15
9	Saline	145 \pm 22	1.53 \pm 0.22
10	Priscoline	125 \pm 14	1.42 \pm 0.19
8	Saline	143 \pm 23	1.48 \pm 0.10
10	Dibenzyline	135 \pm 17	1.33 \pm 0.21
10	Saline	153 \pm 23	1.39 \pm 0.17
11	Atropine	116 \pm 9	1.37 \pm 0.10
10	Saline	135 \pm 8	1.49 \pm 0.08
11	Phenergan	132 \pm 13	1.33 \pm 0.16
10	Saline	135 \pm 8	1.49 \pm 0.08

The effect of histamine depletion on the response of rats to toxin was also investigated. Following the procedures of Riley and West,⁹ rats were treated with Compound 48/80 for 5 days and toxin was injected intravenously on the 6th day. Time to death and lung weights in these rats were the same as in littermates given saline and toxin. Because the rats given 48/80 lost more weight during treatment than did the control animals and because some of them still showed clinical signs of histamine release upon injection of the final dose, another experiment was performed. Rats were given "chronic treatment"⁹ with Compound 48/80 for 16 days; at that time there was no clinical evidence of histamine release upon injection of the drug. On the 17th day, toxin was given to these rats and to littermates injected with doses of saline solution for 16 days. In addition, the food consumption of the latter group was limited so that their weight loss was similar to that of rats given 48/80. The data in Table 2 show that prior treatment with the histamine liberator, Compound 48/80, did not reduce the pulmonary edema caused by toxin.

TABLE 2. EFFECT OF HISTAMINE DEPLETION ON RESPONSE OF RATS TO TOXIN

No. of Rats	Treatment	Mean Weight, grams	Mean Weight Loss, grams	Time to Death, minutes (Mean \pm SD)	Lung Weight, % (Mean \pm SD)
9	48/80 (16 days)	199	-44	120 \pm 8	1.57 \pm 0.11
9	Saline (16 days) (limited food)	182	-54	152 \pm 10	1.56 \pm 0.17
9	None (younger rats)	190		125 \pm 15	1.42 \pm 0.06

A drug with anti-serotonin activity was also tested for effect on the survival time and pulmonary edema in rats given toxin. The data in Table 3 show that UML-491 protected rats against a lethal dose of serotonin injected as long as 3 hours after the final dose of drug. None of these rats showed any signs of illness. Table 4 shows that this treatment with UML-491 did not increase survival time or decrease pulmonary edema in rats given a lethal dose of anthrax toxin.

C. SUBCUTANEOUS AND PERITONEAL VASCULAR LEAKAGE

Postmortem examination of rats killed in 3 to 6 hours by a lethal intraperitoneal dose of toxin revealed, in addition to pulmonary edema, that the peritoneal cavity contained as much as 4 cc of exudate. No peritoneal exudate was found in rats sacrificed at corresponding intervals after the administration of equivalent intraperitoneal doses of nontoxic culture filtrate. In other experiments, rats were injected intraperitoneally with 2.0 cc of toxin, a sublethal dose eliciting only minimal signs of illness when given by this route or with 2.0 cc of nontoxic culture filtrate. At times ranging from 30 minutes to 12 hours afterward, Evans blue was injected intravenously. Five minutes later the rats were sacrificed. There were some animals in which the cavity and viscera were quite moist but from which no fluid could be collected. From 0.3 to 1.2 cc of cloudy fluid was aspirated from the peritoneal cavity of other rats sacrificed at 30, 60, and 80 minutes after injection of toxin. In no case were these samples blue. Samples of fluid from animals sacrificed from 2 to 12 hours after injection of toxin ranged in volume from 0.8 to 3.5 cc and all were blue. The amount of dye that leaked from the circulation increased from 2 to 3 hours after injection of toxin and no further change had occurred by 12 hours. Samples of peritoneal fluid up to 0.8 cc in volume were collected at 30 to 90 minutes from rats given nontoxic culture filtrate and none were blue. At 2 hours and later no fluid could be collected.

Similar experiments were performed to demonstrate vascular leakage elicited by intradermal injection of toxin. Toxin and nontoxic culture filtrate (0.10 cc) were injected into separate sites on the abdominal skin of rats. Dye was injected intravenously at intervals thereafter and the animals were sacrificed 5 minutes later. Examination of rats sacrificed at 1, 1½, 2, and 2½ hours revealed no evidence of dye leakage at the injection sites. The sites of toxin injection in rats sacrificed at 3½ to 8 hours were marked by a clearly discernible area of blue about 1 cm in diameter on the under surface of the skin. There was no area of palpable edema such as that found in the guinea pig 24 hours after intradermal injection of crude toxin. The sites where nontoxic culture filtrate had been injected were not blue.

TABLE 3. EFFECT OF UML-491 IN RATS GIVEN A LETHAL DOSE OF SEROTONIN

	First Dose		Second Dose		Serotonin, mg (intravenous)	No. Killed/ No. Injected
	UML, mg (intraperitoneal)	Time, (minutes)	UML, mg (intravenous)	Time, (minutes)		
1.	10	45-60	3.3	20	10	0/7
2.	10	45-60	3.3	180	10	0/10
3.	-	-	3.3	180	10	0/2
4.	Saline instead of UML in 1 or 2 above				10	21/21

TABLE 4. EFFECT OF UML-491 IN RATS GIVEN A LETHAL DOSE OF TOXIN

No. of Rats	Treatment	Time to Death, minutes (Mean \pm SD)	Lung Weight, % (Mean \pm SD)
9	UML ^a -(60 min)-UML-(15 min)-Toxin	178 \pm 16	1.29 \pm 0.13
9	UML ^a -(60 min)-Toxin-(60 min)-UML		
10	Saline-(60 min)-Toxin-(60 min)-Saline	178 \pm 17	1.27 \pm 0.11

a. First dose 10 mg intraperitoneal; second dose 3.3 mg intravenous.

IV. DISCUSSION

These experiments have shown that the massive pulmonary edema consistently found in rats killed by anthrax toxin develops only after a lag period during which signs of illness are absent. The protein content and electrophoretic analyses of pulmonary exudates collected from rats after death indicate that plasma, per se, had escaped from the pulmonary capillaries. Sublethal doses of toxin also elicited a delayed but prolonged leakage in pulmonary, peritoneal, and subcutaneous vascular beds. Treatment of rats given toxin with atropine, adrenergic blocking drugs, and nicotine did not prolong survival time or prevent pulmonary edema. These experiments thus failed to implicate the autonomic nervous system as a mediator in the action of anthrax toxin. Similar results upon treatment with an antihistamine drug, Compound 48/80, and a potent anti-serotonin compound, along with the fact that vascular leakage is delayed, indicate that histamine and serotonin do not play essential roles in the lethal action. These data suggest that toxin caused pulmonary edema by a direct action on blood vessels but we have no evidence to exclude the possibility that this effect is mediated through other factors elicited by intoxication. Morphologic changes in the capillary endothelium of rats given anthrax toxin were demonstrated by electron microscopy and are described in another report.¹⁰

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<p>The pathogenesis and nature of the pulmonary edema caused by lethal doses of anthrax toxin in Fischer 344 rats were investigated. It was shown that massive pulmonary edema developed only after a lag period during which signs of illness were absent. The protein content and electrophoretic analyses of exudates collected from rats killed by toxin indicated that plasma had escaped from the pulmonary circulation. Following injection of sublethal doses of toxin, a delayed but prolonged vascular leakage in pulmonary, peritoneal, and subcutaneous vascular beds was also demonstrated. Pulmonary edema was not prevented nor was survival time increased by treating rats with drugs inhibiting histamine or serotonin or with drugs blocking the autonomic nervous system at several sites.</p>		

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